

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
8 March 2001 (08.03.2001)

PCT

(10) International Publication Number
WO 01/16359 A2

(51) International Patent Classification⁷: **C12Q 1/527**, (74) Agent: **WOODS, Geoffrey, Corlett; J.A. Kemp & Co., 14 G01N 33/53, A61P 25/00** South Square, Gray's Inn, London WC1R 5LX (GB).

(21) International Application Number: **PCT/GB00/03360**

(22) International Filing Date: 31 August 2000 (31.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9920566.8 31 August 1999 (31.08.1999) GB

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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— Without international search report and to be republished upon receipt of that report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/16359 A2

(54) Title: SCREEN FOR AXON VIABILITY

(57) Abstract: A method for determining the viability of an axon comprises: (i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC); (ii) determining whether sGC is stimulated in the axon; and (iii) determining thereby whether the axon is viable.

SCREEN FOR AXON VIABILITY

Technical field of the invention

This invention relates to methods for assaying for axon viability and to
5 methods for screening for substances which protect axons from loss of viability.

Background to the invention

Axons in CNS white matter become damaged in various debilitating conditions affecting humans, including stroke, trauma and multiple sclerosis (Stys, 10 Trapp et al., 1998). The underlying mechanisms, however, have not been investigated as extensively as those causing damage to grey matter. In part at least, this is attributable to the technical difficulties of studying white matter pathology. The available information on white matter axons has so far come mainly from electrophysiological experiments on the rat isolated optic nerve preparations, in 15 which the degree of recovery of the compound action potential following transient anoxia is used as an index of viability (Stys, 1998). A similar method has been applied to traumatic damage in the spinal cord (Agrawal & Fehlings, 1996).

A quantitative morphometric approach for analysing white matter axon pathology has recently been developed and used to study the mechanisms of rat optic nerve axon degeneration resulting from transient oxygen- and glucose-deprivation (OGD) *in vitro* (Garthwaite et al., 1999). The results suggest a mechanism similar to that proposed to explain anoxic axonal damage (Stys, 1998), namely that excessive influx of Na⁺ through voltage-dependent Na⁺ channels is followed by lethal Ca²⁺ overload of the axoplasm through reversal of the Na⁺-Ca²⁺-exchanger located in the 20 cell membrane. The histological method, however, suffers from the disadvantage of not recording axonal function and so interpretations based purely on morphological 25 criteria may be misleading.

Nitric oxide (NO) functions as a diffusible second messenger molecule in most areas of the central nervous system (CNS). It is generated from L-arginine by 30 NO synthase enzymes, the neuronal isoform of which is functionally and physically associated with the N-methyl-D-aspartate type of glutamate receptor in many brain

areas (Garthwaite & Boulton, 1995; Christopherson & Bredt, 1997). A major mechanism for NO signal transduction is activation of the enzyme soluble guanylyl cyclase (sGC), which causes the formation of cGMP from guanosine 5'-triphosphate (GTP). This pathway appears to mediate many of the physiological actions of NO in the CNS and elsewhere (Ignarro, 1991; Garthwaite & Boulton, 1995; Christopherson & Bredt, 1997; Hobbs, 1997).

Summary of the invention

We have unexpectedly found that the rat optic nerve, a CNS white matter tract which lacks synapses and is composed mainly of glial cells and axons, is capable of generating large quantities of cGMP in response to NO and that this response is confined to the axons. This discrete localization, together with the fact that cGMP formation requires high energy phosphates that are lacking in non-viable tissue, indicated that the response can serve as a sensitive marker for optic nerve axon viability.

The finding that NO leads to cGMP formation in optic nerve cell axons is surprising. Previous evidence has indicated that, in the CNS, the NO-cGMP signalling pathway is primarily associated with synapses, yet synapses are absent from the optic nerve. Also, the neurones giving rise to the optic nerve axons, the retinal ganglion cells, do not appear to react to NO in the same way. In bovine or rat retinae, little or no cGMP immunostaining was observed in these cells in response to NO-donor compounds.

According to the present invention there is thus provided a method for determining the viability of an axon comprising:

- 25 (i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC);
- (ii) determining whether sGC is stimulated in the axon; and
- (iii) determining thereby whether the axon is viable.

30 The invention also provides:

- a method for identifying a substance capable of protecting an axon from loss

of viability comprising:

- (i) contacting an axon with a test substance under conditions that in the absence of the test substance would lead to a decrease in viability;
- (ii) determining the viability of the axon by a method according to any one of the preceding claims; and
- (iii) determining thereby whether the test substance can protect the axon from loss of viability;

- a substance identified by a method for identifying a substance capable of protecting an axon from loss of viability;
- a substance of the invention for use in a method of treatment of the human or animal body by therapy;
- use of a substance of the invention in the manufacture of a medicament for use in the treatment of a condition associated with white matter damage;
- use of a substance of the invention in the manufacture of a medicament for use in the treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease;
- a method of treating a host suffering from a condition associated with white matter damage, which method comprises administering to the host a therapeutically effective amount of a substance of the invention; and
- a method of treating a host suffering from cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease, which method comprises administering to the host a therapeutically effective amount of a substance of the invention.

30 **Brief description of the figures**

Figure 1 shows (a) DEA/NO concentration-response curve for cGMP

accumulation in isolated adult rat optic nerves. (b) Protection of the cGMP response to 100 μ M DEA/NO of OGD-treated optic nerves (shaded columns) by removal of Ca^{2+} (0Ca^{2+}) or Na^+ (0Na^+) or addition of TTX (1 μ M). All 3 treatments significantly restored cGMP level ($P < 0.001$). Data are means \pm S.E.M ($n = 4-9$).

5

Figure 2 shows protection against OGD-induced loss of optic nerve cGMP response to 100 μ M DEA/NO by lamotrigine and analogues. Nerves kept in aCSF throughout are indicated by the open columns; nerves subjected to OGD are shown in shaded columns; * $P < 0.02$; ** $P < 0.0001$ versus OGD alone ($n = 4-12$).

10

Figure 3 shows the histology and cGMP immunohistochemistry in control and OGD-treated optic nerves. (a) Semithin longitudinal section of untreated optic nerve following 5 h incubation. (b,c) cGMP immunostaining in longitudinal frozen sections of nerves incubated without (b) or with (c) DEA/NO for 5 min. (d-f) Semithin sections showing control histology in a transversely-cut optic nerve (d) and cGMP immunostaining in transverse (e) and longitudinal (f) sections of DEA/NO-treated nerves. (g-i) Semithin cross-sections showing the histology of optic nerves subjected to 1 h of OGD in the absence (g) and presence of BW619C89 (100 μ M, h), or 1 μ M TTX (i) followed, in each case, by 90 min recovery in normal aCSF. (j-l) cGMP immunohistochemistry of longitudinal frozen sections from DEA/NO-stimulated nerves previously subjected to 1 h OGD in the absence (j) or presence of BW619C89 (100 μ M, k) or TTX (1 μ M, l). The DEA/NO concentration was 100 μ M in all cases. Key: short arrows, axons; large arrowhead, oligodendrocyte; double small arrowheads, astrocyte soma; open arrows, band of glial cells; curved arrow, astrocyte processes. Scale bar (10 μ m shown in a) applies to all micrographs.

Detailed description of the invention

The present invention provides a method for determining the viability of an axon which consists essentially of the following steps:

- 30 (i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC);

- (ii) determining whether sGC is stimulated in the axon; and
- (iii) determining thereby whether the axon is viable.

This assay for axon viability is significant, as no other simple methods for assessing white matter axon viability are presently available.

In principle the assay for determining the viability of an axon may be carried out to determine the viability of any axon. However, the assay is particularly suitable for determining the viability of white matter axons. White matter is an area of the nervous system, containing abundant myelinated axons and is therefore light in colour. The central nervous system comprising the brain and spinal cord and the peripheral nervous system both contain white matter and axons from these sources may be used in the assay of the invention. Axons from the optic nerve are particularly suitable.

In principle the assay may be carried out using a single axon. However, in practice it is more convenient to use more than one axon in a single assay. Typically, a population of axons, for example a nerve, is used. The viability determined when more than one axon is used will represent an average viability for the population of axons used.

In viable axons, NO activates sGC, leading to an increase in cGMP formation, which in turn leads to the modulation of the activity of a number of cGMP targets. A viable axon may thus be identified by determining whether this pathway is functional in that axon. The activity of sGC before and after contacting an axon with a substance capable of stimulating sGC may be determined in order to determine whether sGC activity is stimulated, thereby to determine whether the axon is viable.

Any suitable format may be used for carrying out the assay of the invention. Generally, the assay is carried out *ex vivo* and under physiologically acceptable conditions; that is, under conditions that would be expected to support axon survival. It will often be convenient to carry out the assay in an aqueous medium, for example a physiologically acceptable buffer.

Typically, the assay is initiated by contacting an axon with a substance that is capable of stimulating sGC. Such a substance is generally one which under normal physiological conditions is capable of activating sGC in a viable axon. Suitable

activators of sGC include nitric oxide (NO), 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), carbon monoxide (CO) or YC-1 and CO. A combination of YC-1 and CO is a very effective activator of sGC.

Stimulators of sGC may be supplied in any way. For example, NO may be supplied in the form of an NO donor. This is particularly suitable if the assay is carried out in an aqueous environment. Suitable NO donors include organic nitrates (eg. glyceryl trinitrate), nitrites (eg. amyl nitrite), inorganic nitroso compounds (eg. sodium nitroprusside), sydnonimines (eg. molsidomine, 3-morpholinosydnonimine), S-nitrosothiols (eg. S-nitroso-L-cysteine, S-nitrosoglutathione, S-nitroso-N-acetyl-L-cysteine, S-nitroso-N-acetyl-DL-penicillamine) and 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO). Such donors may be added to a final concentration of between for example 10nM to 300μM. The half-life of the above mentioned donors vary. The half-life of DEA/NO is, for example, approximately 2 minutes. Donors with shorter half-lives, for example 1 to 5 minutes are preferred and those with half-lives of 2 to 3 minutes are most preferred.

Determining whether sGC is stimulated may be carried using any suitable method. Typically sGC activity is determined before and after contacting an axon with a substance capable of stimulating sGC. The activity of sGC can be determined directly. It is generally most convenient to do this by measuring the production of cGMP by sGC. For example, by measuring the conversion of radiolabelled GTP into cGMP. Alternatively or additionally, a pH sensitive probe may be used to determine sGC activity, as H⁺ ions are also produced by the enzymatic reaction catalysed by sGC. A further method for measuring the activity of sGC is to use a fluorescent tag on the sGC enzyme. In such a method sGC is modified using recombinant DNA techniques so that the sGC comprises a fluorescent polypeptide domain. The fluorescent properties of the resulting sGC: fluorescent polypeptide enzyme change depending on the activity of the enzyme.

It is most convenient to determine whether sGC is stimulated by measuring cGMP levels before and after contacting an axon with a substance that is capable of stimulating sGC. The production of cGMP may be determined by any suitable technique known to those skilled in the field. For example, radioimmunoassays,

enzyme-linked immunoassays (ELISA) and immunohistochemistry may be used. If radioimmunoassays or ELISA are used, typically the total protein content of the tissue is also assayed. In that way the amount of cGMP in a sample can be expressed per amount of protein. Radioimmunoassays, ELISA and immunohistochemistry may all be carried out using anti-cGMP antibodies. Any suitable antibodies may be used. For example, suitable antibodies for use in immunohistochemistry are described in De Vente *et al.* (1987). The above techniques are all well known to those skilled in the art.

cGMP is broken down in cells by the action of phosphodiesterases (PDEs).

Therefore, the rate of cGMP accumulation is the difference between its rate of formation by sGC and its rate of destruction by PDEs and if PDE activity is high, cGMP accumulation may not be observed. Thus, PDE inhibitors, for example non-selective PDE inhibitors such as 3-isobutyl-1-methylxanthine (IBMX), may also be added to the assay. In the presence of such inhibitors the rate of cGMP accumulation is equal to the rate of cGMP formation.

The activity of sGC may also be determined indirectly by measuring, for example, the activity of a target of cGMP. Thus, for a viable axon sGC stimulation may be determined by measuring any modulation in the activity of a cGMP target. A number of cGMP targets are known. For example, cGMP activates cGMP dependent protein kinase as well as ion channels. Additionally, the activities of phosphodiesterases are modulated in response to cGMP. Measurement of any of these targets may be used to, indirectly, determine whether sGC is stimulated.

Appropriate control experiments may be carried out when performing the assay of the invention. For example, the assay will be carried out in both the absence and presence of a substance capable of stimulating sGC. Additionally, if cGMP increase or modulation of a cGMP target are measured, the involvement of sGC stimulation may be confirmed by carrying out the assay in the presence of an inhibitor of sGC, for example 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. If sGC is involved in the elevation of cGMP levels in response to NO stimulation, the presence of an sGC inhibitor will reduce the cGMP response observed in the absence of that inhibitor.

A non-viable axon may be assayed to determine whether any sGC stimulation occurs in that axon. An axon may be rendered non-viable by subjecting it to for example, oxygen deprivation and/or sugar, eg. glucose, deprivation. Typically, it is preferable to use conditions under which irreversible damage to the axon occurs. For 5 example, incubating nerves in a medium with no glucose and gassed with 5% CO₂ in N₂ for 1 hour causes irreversible damage to the majority of axons so incubated (Garthwaite *et al.*, 1999).

Other types of cell known to exhibit sGC stimulation and increase in cGMP formation in response to NO may be used as positive controls. For example vascular 10 endothelial cells show an increase in cGMP formation on stimulation with NO and could therefore be used as positive control in the assay.

Generally, a viable axon is one which shows greater sGC stimulation than that shown by a non-viable axon. Typically, a viable axon will show an increase in sGC activity of at least 2-fold that shown by a non-viable axon. More preferably, a 15 viable axon will show an increase in sGC activity of at least 25-fold, more preferably 50-fold that shown by a non-viable axon.

Similarly, if modulation of activity of a cGMP target is used to measure sGC stimulation, a viable axon is one which shows greater modulation of activity of a cGMP target than that shown by a non-viable axon.

If cGMP generation is used as a measure of sGC stimulation, a viable axon is generally one which shows a greater increase in cGMP generation than that shown by a non-viable axon. Typically, a viable axon will show an increase in cGMP 20 generation of at least 2-fold that shown by a non-viable axon. More preferably, a viable axon will show an increase in cGMP generation of at least 25-fold, more 25 preferably 50-fold that shown by a non-viable axon.

The magnitude of the sGC stimulation observed may depend on the concentration of sGC stimulator present in the assay. Therefore greater sGC stimulation may be observed when higher concentrations of sGC stimulator are used. A viable axon will preferably show sGC stimulation at low concentrations of sGC 30 stimulator.

The invention also provides a method of identifying a substance capable of

protecting an axon from loss of viability, a "protectant". Thus, substances may be identified which preserve axon viability under conditions that would typically lead to axon damage or axon death. Substances identified by such methods may be useful in the prevention and/or treatment of conditions in which damage to or death of axons, 5 in particular CNS white matter axons, is implicated.

Any suitable format may be used for identifying a substance capable of protecting an axon from loss of viability. The assay is, however, typically carried out in an aqueous medium and preferably in a single well of a plastics microtitre plate, so that high through-put screening for protectants may be carried out.

10 Typically an axon is contacted with a test substance under conditions that, in the absence of the test substance, would lead to a reduction in viability of that axon. Suitable conditions are described above. The viability of an axon may be determined using the viability assay of the invention and this will allow the ability of a test substance to prevent loss of viability to be ascertained.

15 Suitable control experiments may be carried out. For example, the method may be carried out in the absence of a test substance in order to determine any basal level of sGC stimulation for non-viable axons. Positive control assays may be carried out using the known neuroprotectants, lamotrigine, BW619C89 and BW1003C78 (Xie *et al.*, 1995; Xie and Garthwaite, 1996; Meldrum *et al.*, 1992)

20 Combinatorial libraries, defined chemical entities, peptide and peptide mimetics, oligonucleotides and natural product libraries may be screened for activity as protectants in assays such as those described above. The candidate substances may be chemical compounds. The candidate substances may be used in an initial screen of, for example, ten substances per reaction, and the substance of these 25 batches which show inhibition tested individually. Suitable candidate substances include antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimaeric antibodies and CDR-grafted antibodies).

20 A substance which is capable of protecting an axon from a loss of viability, a "protectant", is one which causes a measurable increase in axon viability in the method described above. Preferred substances are those cause an increase in axon viability of at least 10%, at least 25%, at least 50%, at least 100% at least 200%, at

least 500%, at least 1000%, at least 50000%, at least 100000% at a concentration of the protectant of $1\mu\text{g ml}^{-1}$, $10\mu\text{g ml}^{-1}$, $100\mu\text{g ml}^{-1}$, $500\mu\text{g ml}^{-1}$, 1mg ml^{-1} , 10mg ml^{-1} , 100mg ml^{-1} . The percentage increase represents the percentage increase in axon viability in a comparison of assays in the presence and absence of the test substance.

5 Any combination of the above mentioned degrees of percentage increase in axon viability and concentration of protectant may be used to define a protectant of the invention, with greater increase in axon viability at lower concentrations of protectant being preferred.

10 Candidate protectants which show activity in assays such as those described above can then be tested in *ex vivo* models and *in vivo* models. A suitable *ex vivo* model involves dosing an animal with a neuroprotective agent. After a suitable time for absorption and brain penetration of the agent, the animal is killed. The decapitated head is left at normal body temperature for a given interval (eg. 1h) and then the optic nerves are taken out, incubated in vitro and assayed for viability.

15 Suitable *in vivo* models include traumatic damage to the spinal cord (which damages white matter). Animal models exist for the majority of the indications given below and are well known to those skilled in the art.

20 Protectants identified by the screening procedures described above may be used to treat any condition associated with white matter damage. Conditions associated with white matter damage include cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, viral infections (eg. human immunodeficiency virus), alcohol abuse, cerebral malaria and motoneurone disease. Additionally, protectants of the invention may be used in the manufacture of a medicament for use in the treatment of one of the above mentioned indications. The condition of a patient suffering from any of the above mentioned conditions can therefore be improved by administration of such a protectant of the invention. A therapeutically effective amount of a protectant of the invention may be given to a human patient in need thereof.

25 Protectants of the invention may be administered in a variety of dosage forms. Thus, they can be administered orally, for example as tablets, troches, lozenges,

aqueous or oily suspensions, dispersible powders or granules. The protectants may also be administered parenterally, either subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. The protectants may also be administered as suppositories. A physician will be able to 5 determine the required route of administration for each particular patient.

The formulation of a protectant for use in the treatment of a condition associated with white matter damage will depend upon factors such as the nature of the exact protectant, whether a pharmaceutical or veterinary use is intended, etc. A protectant may be formulated for simultaneous, separate or sequential use.

10 A protectant is typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the active compound, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, 15 talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, gum arabic, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescent mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in 20 general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tabletting, sugar-coating, or film-coating processes.

25 Liquid dispersions for oral administration may be syrups, emulsions or suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may 30 contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired,

a suitable amount of lidocaine hydrochloride.

Solutions for intravenous administration or infusion may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

5 A therapeutically effective amount of a protectant is administered to a patient. The dose of a protectant may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage 10 for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg of body weight, according to the activity of the specific protectant, the age, weight and conditions of the subject to be treated, the type and severity of the degeneration and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

15 The following Example illustrates the invention.

Example

Materials and methods

Optic nerve preparation

Nerves (about 9 mm long) were excised from adult Wistar rats (240-280 g) after decapitation. They were incubated in Erlenmeyer flasks (50 ml capacity) containing 20 ml of an artificial CSF (aCSF) solution composed of (mM): NaCl (120) KCl (2.0), CaCl₂ (2.0), NaHCO₃ (26), KH₂PO₄ (1.18), MgSO₄ (1.19) and glucose (11), 25 continuously gassed with 95% O₂/5% CO₂. The flasks were held in a shaking water bath at 37°C. For the Ca²⁺-free medium, ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (1 mM) was substituted for CaCl₂ and for the Na⁺-free medium, 120 mM choline chloride and 26 mM choline bicarbonate replaced NaCl and NaHCO₃ respectively.

Oxygen and glucose deprivation.

After 1-2 h preincubation in aCSF, test nerves were transferred into aCSF lacking glucose and gassed with 5% CO₂ in N₂ for 1 h, a period shown previously to result in irreversible damage to the majority of axons (Garthwaite *et al.*, 1999). Afterwards, the nerves were given a 90 min recovery period in normal aCSF. Modified aCSF and putative axonoprotective compounds were present from 15 min before until 15 min after OGD.

cGMP accumulation

Nerves, with or without a preceding 1 h exposure to OGD (plus 90 min recovery) were exposed to the nitric oxide (NO) donor, DEA/NO (2,2-diethyl-1-nitroso-oxyhydrazine) for 5 min. They were then inactivated in boiling hypotonic buffer and their protein and cGMP contents measured using the automated Lowry method and radioimmunoassay, respectively, as described (Garthwaite & Garthwaite, 1987). The general phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 1 mM) was added 10 min before the exposure to the NO donor, except where indicated. Results are given as means ± SEM and were evaluated using the unpaired Student's *t*-test (2-tailed), P < 0.05 being considered significant.

Histology and cGMP Immunohistochemistry

Conventional histology was carried out on semithin sections of resin-embedded nerves as described previously (Garthwaite *et al.*, 1999). For cGMP immunohistochemistry, nerves, with or without various treatments (as described in the text) were fixed in ice-cold, freshly-depolymerised paraformaldehyde (4%) in 0.1 M phosphate buffer (pH 7.4) for 2 h, processed as described before (Southam & Garthwaite, 1993), and then frozen on a cryostat chuck and sectioned at 10 µm intervals. Some nerves were embedded in resin (Durcupan) using conventional methods and cut into 1 µm thick sections. cGMP immunostaining was conducted using a sheep anti-cGMP antibody (Tanaka *et al.*, 1997). Briefly, the sections were incubated with primary antibody (1:80,000) overnight at 4°C. They were then incubated at room temperature with rabbit biotinylated anti-sheep antibody (1:1000; 1 h) followed by Vector stain ABC elite kit (30 min) and then 3,3'-diaminobenzidine

(4 min). Counterstaining was carried out using Mayer's haemalum for 15 s. The 1 μ m thick resin-embedded sections were etched with 1:1 mixture of ethanol and saturated sodium hydroxide in ethanol for 5 min before immunohistochemistry; these sections were counterstained with Mayer's haemalum for 5 min.

5

Materials

The sheep anti-cGMP antibody was a kind gift from Dr. J. de Vente (Maastricht, Netherlands). Secondary antibodies and the ABC kit were purchased from Vector laboratories (Orton Southgate, Peterborough, UK). DEA/NO was from Alexis Corporation (Bingham, Nottingham, UK) or RBI (through Semat Technical UK Ltd., St. Albans, Herts, UK). Tetrodotoxin was from Latoxan Laboratories (Rosans, France). Lamotrigine, BW619C89 and BW1003C87 were supplied by the Wellcome Research Laboratories (Beckenham, Kent). Other chemicals were from Sigma-Aldrich (Poole, Dorset, UK), BDH/Merck (Poole, Dorset, UK) or Tocris-Cookson (Bristol, UK).

Results

Basal cGMP levels in the rat optic nerves averaged 1.06 ± 0.14 pmol/mg protein ($n = 4$) and the levels were 3-fold higher in presence of the non-selective phosphodiesterase inhibitor, IBMX (1 mM; 3.55 ± 0.36 pmol/mg protein; $n = 8$). To test the ability of NO to elevate cGMP levels in this tissue, the NO-donor DEA/NO, which dissociates with a half-life of about 2 min (Morley & Keefer, 1993) was used. Exposure of the nerves for 5 min to DEA/NO (10 nM - 300 μ M), in the presence of IBMX, resulted in concentration-dependent accumulation of cGMP to levels that were ultimately more than 50-fold higher than in the unstimulated tissue (Fig. 1a). Half-maximal effects occurred at about 10 μ M DEA/NO. The inhibitor of NO-stimulated soluble guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (Garthwaite *et al.*, 1995), at a concentration of 3 μ M (10 min preincubation), reduced the cGMP response to 100 μ M DEA/NO from 219 ± 23 to 32 ± 1 pmol/mg protein ($n = 4$) confirming the involvement of this enzyme. In the absence of IBMX, maximal cGMP accumulation (with 100 μ M DEA/NO), instead of being more than 200

pmol/mg protein, was only 32 ± 3 pmol/mg protein ($n = 4$), implying a high endogenous phosphodiesterase activity.

Conventional histology of resin-embedded nerves showed that, under control conditions, axons and glial cells (astrocytes and oligodendrocytes) were well preserved in incubated optic nerves (Fig. 2a,d), in agreement with previous findings (Waxman *et al.*, 1992; Garthwaite *et al.*, 1999). To locate the sites of cGMP accumulation, immunohistochemistry was used. In frozen sections from unstimulated nerves (incubated with IBMX), no immunostaining was observed (Fig. 2b). In contrast, exposure to 100 μ M DEA/NO (in the presence of IBMX) for 5 min produced powerful staining that was apparently restricted to axons (Fig. 2c). Higher resolution immunohistochemical staining, carried out on semithin sections from resin-embedded nerves, confirmed the staining to be in axons, with no detectable labelling of myelin or glial cells (Fig. 2e,f).

When optic nerves were subjected to 1 h of OGD followed by 90 min recovery in normal aCSF, histology showed abundant axonal swelling (Fig. 2g). The biochemically-measured cGMP response to DEA/NO (100 μ M) in nerves previously subjected to OGD was reduced by about 80% (Fig. 1b & 3) and cGMP immunohistochemistry of such nerves showed a marked loss of labelled axons; although there remained a few that stained normally (Fig. 2j).

To further examine the validity of the cGMP response as a marker of axon viability, manoeuvres found previously to reduce or eliminate anoxia-induced loss of the optic nerve compound action potential (Stys, 1998) or OGD-induced axon pathology (Garthwaite *et al.*, 1999) were tested. Complete preservation of the cGMP response was achieved if OGD was imposed in Ca^{2+} -free aCSF or in the presence of the voltage-dependent Na^+ channel inhibitor, tetrodotoxin (TTX, 1 μ M); Na^+ -free aCSF was less effective, affording only 60% protection (Fig. 1b). Control experiments showed that the cGMP response of nerves exposed to Ca^{2+} -free or Na^+ -free aCSF, or TTX, for the same intervals (but without OGD) were normal ($n = 4$, results not shown). When examined under the microscope, TTX prevented OGD-induced axonopathy (Fig. 2i) and, in parallel, OGD-induced loss of cGMP immunostaining of the axons following exposure to DEA/NO (Fig. 2l). Similar

results were found with Ca²⁺-free solution (results not shown).

Anoxic damage to optic nerve, assayed using electrophysiology, has been shown to be lessened in the presence of certain antiepileptic drugs (e.g. phenytoin and carbamazepine), local anaesthetics and antiarrhythmic agents (Stys, 1998). The efficacy of these measures is explained by their capacity to block voltage-dependent Na⁺ channels. The newer antiepileptic drug, lamotrigine, and the structurally related molecule, BW619C89, block Na⁺ channels in a use- and voltage-dependent manner (Xie *et al.*, 1995; Xie & Garthwaite, 1996) and are neuroprotective towards grey matter *in vivo* (Taylor & Meldrum, 1995; Urenjak & Obrenovitch, 1996). Hence, these compounds, and the structurally-related neuroprotectant, BW1003C87 (Meldrum *et al.*, 1992), were tested for their ability to protect the optic nerve against OGD using histology and the NO-stimulated cGMP accumulation.

The compound BW619C89 provided concentration-dependent protection against OGD-induced loss of the cGMP response (Fig. 3), the half-maximal effect being observed at about 6 µM. At the highest concentrations (30-100 µM), the response amplitude was not significantly different from that of control nerves that had not been subjected to OGD. Substantial, though incomplete, protection was also achieved with BW1003C87 (30 µM; 60% protection) and lamotrigine (100 µM; 40% protection) (Fig. 3). On their own, none of the 3 compounds had an adverse effect on the ability of nerves to produce cGMP in response to DEA/NO (Fig. 3 and results not shown). Histology and cGMP immunohistochemistry correlated well with the biochemical results: for example, BW619C89 (30 µM) protected the axons from OGD-induced pathology (Fig. 2h) and loss of axonal cGMP immunostaining (Fig. 2k).

Discussion

The existence of the NO receptor, soluble guanylyl cyclase, in optic nerve was not previously known. Signalling by NO through this mechanism has, however, been described in many other tissues and it appears to be the principal pathway through which physiological NO signalling occurs (Ignarro, 1991; Garthwaite & Boulton, 1995; Christopherson & Bredt, 1997; Hobbs, 1997). The finding that NO

led to cGMP formation specifically in optic nerve axons is surprising for two reasons. First, previous evidence had indicated that, in the CNS, the NO-cGMP signalling pathway is primarily associated with synapses, particularly those mediating glutamatergic neurotransmission (Garthwaite & Boulton, 1995; 5 Christopherson & Bredt, 1997), yet synapses are absent in the optic nerve. Second, the neurones giving rise to the optic nerve axons, the retinal ganglion cells, do not appear to react to NO in the same way because, in bovine or rat retinae, little or no cGMP immunostaining was observed in these cells in response to NO-donor compounds (Gotzes *et al.*, 1998). This may indicate that NO-sensitive guanylyl 10 cyclase is preferentially targeted to the axons rather than to the somatodendritic regions of these particular neurones. Judging by the large enhancement of NO-induced cGMP accumulation brought about by IBMX, the axons are also likely to be rich in phosphodiesterase activity, supporting the possibility that the expression of the guanylyl cyclase there has functional relevance.

Concerning possible sources of endogenous NO in the optic nerve, there is histochemical evidence that guinea-pig optic nerve astrocytes contain an NO synthase enzyme (Qi & Guy, 1996) but we have been unable to detect the endothelial, neuronal or the inducible NO synthase isoforms in glia or axons of the normal rat optic nerve by immunohistochemistry. Staining for the endothelial 15 isoform in endothelial cells themselves, however, was clearly observed (unpublished observations). Thus, NO derived from endothelial cells might constitute the normal effector for the stimulation of cGMP accumulation in optic nerve axons. If so, this would constitute an unusual pathway for intercellular signalling by NO. Additional sources of NO may be present in pathological conditions since, in human 20 glaucomatous patients, the three different NO synthase isoforms are apparently expressed in optic nerve glia (Neufeld *et al.*, 1997), raising the possibility that this pathway is relevant to disorders of optic nerve function in humans. Understanding the functional consequences of cGMP formation in the axons awaits investigation 25 but, in pilot experiments, we have observed that NO-donors elicit a depolarising response from the optic nerve, suggesting a possible action on axonal ion channels 30 (unpublished observation).

cGMP is synthesised from GTP which exists in equilibrium with adenosine 5'-triphosphate (ATP) intracellularly (Voet & Voet, 1995); consequently, non-viable tissue, lacking high energy phosphates, is unable to generate cGMP in this manner, even if the synthetic enzyme should remain intact. The dependence of the cGMP response on cellular viability has been exploited previously for the identification of the sources and targets of NO in the cerebellum (Garthwaite & Garthwaite, 1987). The significant features of the response in the optic nerve were first, its apparently exclusive location in axons and secondly its magnitude, the two together making NO-induced cGMP accumulation a sensitive marker for optic nerve axon viability.

Accordingly, in optic nerves previously subjected to 1 h of OGD, the cGMP response was only 17% of its value in control nerves. The residual cGMP elevation was attributable (on the basis of immunohistochemistry) to the survival and normal behaviour of a subpopulation of axons (seemingly distributed randomly), as opposed to a generalised reduction in the ability of axons to generate cGMP. The extent of functional axonal loss recorded with this technique is in excellent agreement with that recorded electrophysiologically, in which 1 h of OGD caused an 80% loss of the optic nerve compound action potential (Fern *et al.*, 1998). Moreover, the various procedures that were found previously to protect optic nerve axons from OGD to differing extents, as judged by a morphometric method (Garthwaite *et al.*, 1999), all had quantitatively very similar effects on the level of NO-induced cGMP accumulation. The correspondence in the readout of two independent methods (one based on histology, the other on function) lends strong support to their reliability for assessing optic nerve axon pathology.

Interpretation of the findings with respect to the mechanism of OGD-induced damage follows that proposed from very similar findings made previously using the quantitative morphometric method (Garthwaite *et al.*, 1999). In brief, the findings indicate that the damage is dependent on the activity of voltage-dependent Na⁺ channels and an influx of Ca²⁺ into the axoplasm and are consistent with a mechanism proposed to account for anoxia-induced damage, namely influx of Na⁺ followed by reversal of the Na⁺-Ca²⁺-exchanger leading to a Ca²⁺ overload of the axoplasm (Stys, 1998). The lesser protective efficacy of Na⁺-free aCSF may be

explained by this manoeuvre itself causing influx of Ca^{2+} which could sum with Ca^{2+} coming in via routes other than the Na^+ - Ca^{2+} -exchanger during OGD (Stys & Lopachin, 1998).

Two of the pharmacological agents tested, lamotrigine and BW619C89, have been shown by detailed electrophysiological analysis to be use- and voltage-dependent blockers of voltage-dependent Na^+ channels in central neurones and in cell lines expressing type II Na^+ channels (Xie *et al.*, 1995; Xie & Garthwaite, 1996). The third compound, BW1003C87, is likely to have a similar action since it has a closely related structure and it inhibits glutamate release from brain tissue exposed to the Na^+ -channel opener, veratrine, but not the release induced by raised K^+ (Meldrum *et al.*, 1992). All three compounds protect grey matter from ischaemia *in vivo* (Taylor & Meldrum, 1995; Urenjak & Obrenovitch, 1996). In the present study, BW619C89 protected the axons with a potency and efficacy very similar to those registered by morphometric assay (Garthwaite *et al.*, 1999); the degree of protection achieved by the other compounds, at concentrations shown to be maximally effective, also matched those reported by morphometric assay (Garthwaite *et al.*, 1999). The explanation for the differential protective efficacies of the three structurally-similar molecules towards optic nerve axons (BW619C89>BW1003C87>lamotrigine) awaits investigation but it may relate to a differential blockade of the non-inactivating axonal Na^+ channels that appear responsible for much of the Na^+ influx, at least under conditions of anoxia (Stys *et al.*, 1993). Molecules like BW619C89 which appear able to afford a high degree of protection towards both white matter axons and grey matter subjected to ischaemia-like insults, should, in principle, offer superior treatment for conditions such as stroke than strategies (e.g. glutamate receptor blockade) only capable of protecting grey matter.

In conclusion, in the rat optic nerve, the axons selectively and richly express functional NO receptor protein, enabling them to generate large amounts of cGMP in response to NO. While the functional implications of this response remain to be defined, its existence provides a novel, simple and reliable method for quantitatively assessing axonal viability that is likely prove valuable in studies of the pathogenesis of axonal damage and for assessing axonoprotective measures.

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CLAIMS

1. A method for determining the viability of an axon comprising:
 - (i) contacting the axon with a substance that is capable of stimulating soluble guanylate cyclase (sGC);
 - (ii) determining whether sGC is stimulated in the axon; and
 - (iii) determining thereby whether the axon is viable.
- 5 2. A method according to claim 1, wherein the axon is a white matter axon.
- 10 3. A method according to claim 2, wherein the white matter axon is from the optic nerve, the brain or the spinal cord.
4. A method according to any one of the preceding claims, wherein step (i) is carried out by contacting the axon with nitric oxide (NO), 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), carbon monoxide (CO) or YC-1 and CO.
- 15 5. A method according to claim 4 wherein NO is provided in the form of an NO donor.
6. A method according to claim 5, wherein the NO donor is 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO).
- 20 7. A method according to any one of the previous claims, wherein step (ii) is carried out by determining whether cGMP generation by the axon increases.
8. A method according to claim 8, wherein the generation of cGMP is determined by radioimmunoassay or immunocytochemistry.
- 25 9. A method according to claim 7 or 8, wherein a viable axon is one which shows a greater increase in cGMP generation than that shown by a non-viable axon.
10. A method according to claim 9, wherein the increase in cGMP production is at least 2-fold that shown by a non-viable axon.
11. A method for identifying a substance capable of protecting an axon from loss of viability comprising:
 - (i) contacting an axon with a test substance under conditions that in the absence of the test substance would lead to a decrease in viability;

- (ii) determining the viability of the axon by a method according to any one of the preceding claims; and
- (iii) determining thereby whether the test substance can protect the axon from loss of viability.

- 5 12. A substance identified by a method according to claim 11.
13. A substance according to claim 12 for use in a method of treatment of the human or animal body by therapy.
14. A substance according to claim 13 for use in a method of treatment of a condition associated with white matter damage.
- 10 15. A substance according to claim 14 for use in a method of treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease.
- 15 16. Use of a substance according to claim 11 in the manufacture of a medicament for use in the treatment of a condition associated with white matter damage.
- 20 17. Use of a substance according to claim 11 in the manufacture of a medicament for use in the treatment of cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease.
- 25 18. A method of treating a host suffering from a condition associated with white matter damage, which method comprises administering to the host a therapeutically effective amount of a substance according to claim 11.
- 30 19. A method of treating a host suffering from cerebral ischaemia, epilepsy, multiple sclerosis, spinal cord ischaemia, glaucoma, age-related neuropathology, trauma to the head or spinal cord, diabetes, a viral infection, alcohol abuse, cerebral malaria or motoneurone disease, which method comprises administering to the host a therapeutically effective amount of a substance according to claim 11.

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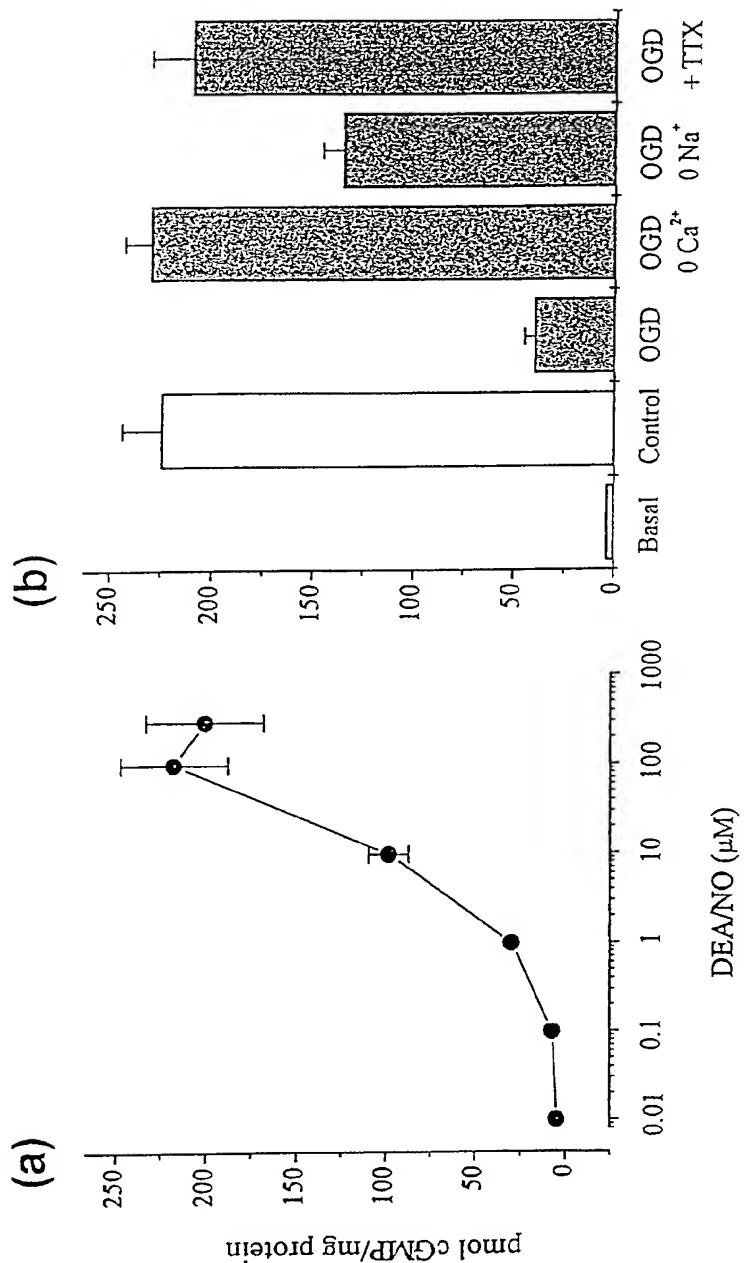


Figure 1

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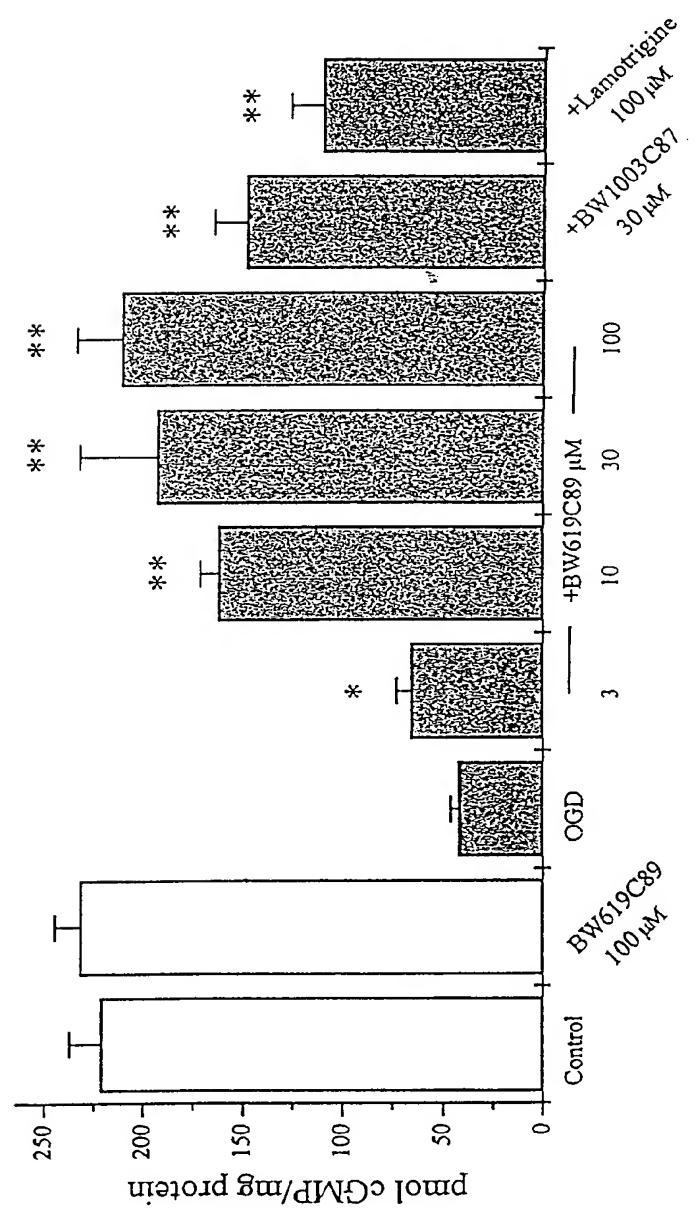


Figure 2

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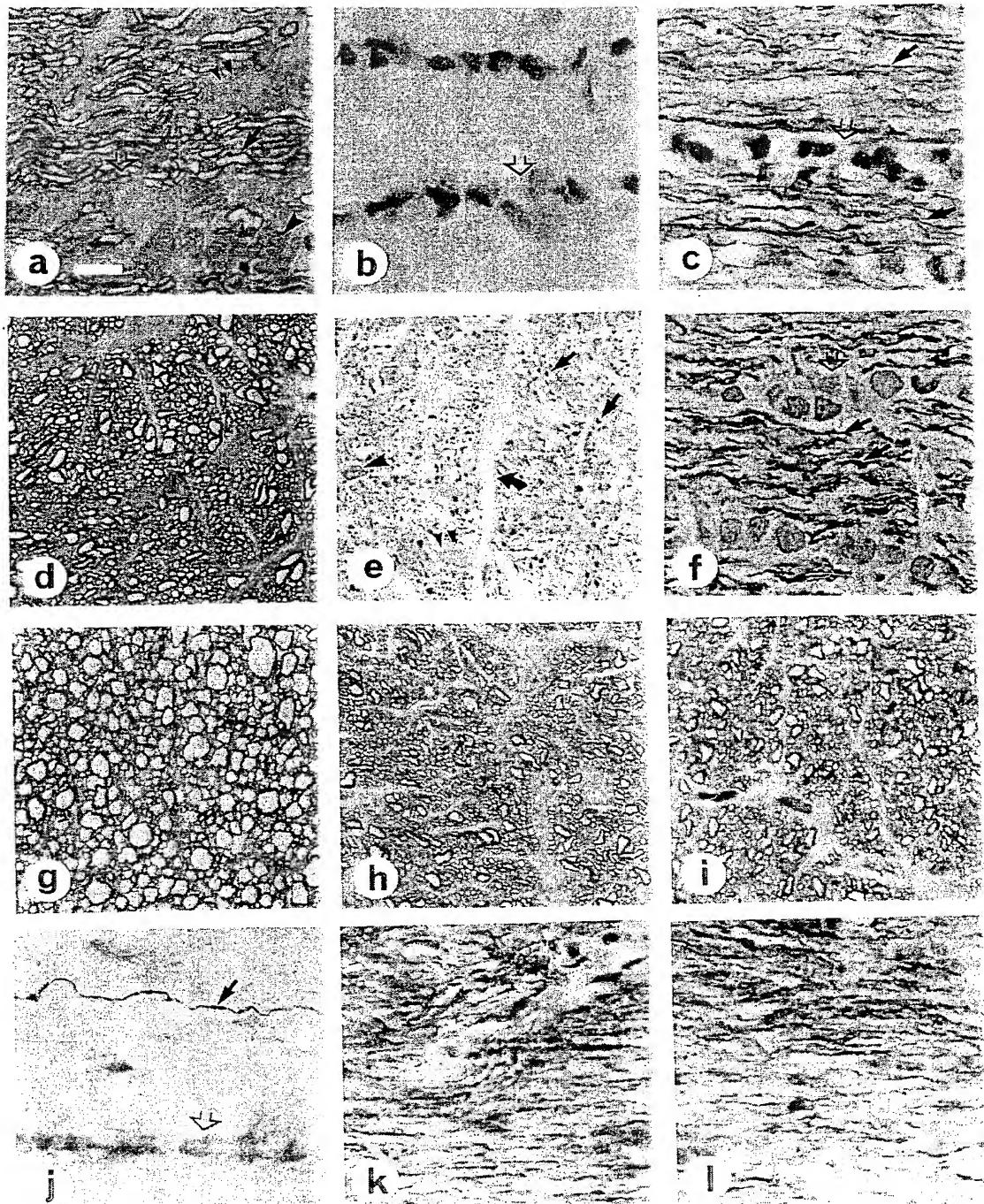


Figure 3